

UNIVERSIDADE ESTADUAL DO CENTRO-OESTE, UNICENTRO-PR

**COMPARAÇÃO DOS TRANSCRIPTOMAS DA
CARTILAGEM E DA PLACA DE CRESCIMENTO DO
FÊMUR DE FRANGOS DE CORTES NORMAIS E
AFETADOS COM NECROSE DA CABEÇA DE FEMUR**

DISSERTAÇÃO DE MESTRADO

IARA GOLDONI

GUARAPUAVA-PR

2021

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Dissertação apresentada a Universidade Estadual do Centro-Oeste, como parte das exigências do Programa de Pós-Graduação em Ciências Veterinárias, área de concentração em Saúde e Produção Sustentável, para obtenção do título de mestre.

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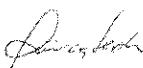
COMPARAÇÃO DOS TRANSCRIPTOMAS DA CARTILAGEM E DA PLACA DE CRESCIMENTO DO FÊMUR DE FRANGOS DE CORTES NORMAIS E AFETADOS COM NECROSE DA CABEÇA DE FEMUR

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RESUMO

Iara Goldoni. Comparação dos transcriptomas da cartilagem e da placa de crescimento do fêmur de frangos de corte normais e afetados com necrose de cabeça de fêmur.

Resumo: A separação da cabeça femoral (FHS) é geralmente uma condição subclínica caracterizada pelo descolamento da cartilagem articular do osso e é considerada a fase inicial da necrose de cabeça de fêmur (FHN), também conhecida como condronecrose bacteriana com osteomielite (BCO), um dos principais problemas locomotores que afetam a produção avícola. Neste estudo foi realizada uma análise abrangente identificando o perfil de expressão gênica compartilhado entre a cartilagem articular femoral (AC) e a placa de crescimento (GP) da cabeça do fêmur de frangos de corte normais e afetados com FHS aos 35 dias de idade. Processos biológicos e genes comuns entre AC e GP relacionados à FSH foram prospectados por meio da análise de sequenciamento de RNA. Diferindo dos estudos anteriores que investigaram a doença considerando somente o tecido ósseo, aqui foram analisados conjuntamente, dados contendo 8 transcriptomas da AC e a GP de frangos de corte normais e 8 afetados por FHS. Foram identificados 36 genes diferencialmente expressos (DE) entre os grupos normais e afetados por FHS compartilhados entre AC e GP, envolvidos em processos biológicos de transporte de íons e resposta imune relacionados com esta condição. Ao comparar os genes DE comuns com outros conjuntos de dados públicos associados à FHN, foram identificados genes possivelmente envolvidos com a doença em diferentes espécies, como humanos. Neste trabalho, os genes *SLC4A1*, *RHAG*, *ANK1*, *MKNK2*, *SPTB*, *ADA*, *C7* e *EPB420* foram considerados possíveis candidatos ao desenvolvimento de FHS/FHN/BCO, contribuindo para a caracterização de processos biológicos e o entendimento da etiologia desta condição.

Palavras-chave: necrose de cabeça de fêmur, RNA-Seq, galinha,

ABSTRACT

Iara Goldoni. Comparison of articular cartilage and growth plate transcriptomes from normal and femoral head separation affected broilers.

Abstract: The Femoral Head Separation (FHS) is usually a subclinical condition characterized by the detachment of articular cartilage from the bone. This condition is closely related to Femoral Head Necrosis (FHN), one of the main locomotor problems that affect the poultry production. Therefore, in this study, a comprehensive analysis identifying shared expression profiles, biological processes (BP) and variants related to FHS and subsequent FHN in chickens was performed through RNA sequencing analysis. To this, two datasets containing 8 normal and 8 FHS-affected transcriptomes from broilers femoral articular cartilage (AC) and growth plate (GP) were analyzed. Furthermore, we have compared the differential gene expression found in FHS-chickens with other datasets available in public databases, trying to clarify shared genes and biological processes related to FHN in different species. A total of 36 differentially expressed (DE) genes were shared between AC and GP tissues, where more than 90% was upregulated in the FHS-affected broilers. Out of those, 23 genes were enriched in BP related to ion transport, translation factors and immune response. When the chicken DE genes were compared to other FHN datasets, 8 genes were shared among the evaluated species with normal and FHN affected samples: *SLC4A1*, *EPB42*, *ANK1*, *SPTB*, *CCL26*, *ADA*, *SLC25A37* and *MKNK2*. This study found DE genes between normal and FHS-affected broilers common to both

analyzed tissues, showing shared mechanisms involved with this condition in chickens. Comparing the AC and GP DE genes with other datasets related to FHN allowed us to identify genes that are possibly involved with FHN across different species. We were able to find strong candidate genes related to FHS and FHN in chickens, such as *SLC4A1*, *RHAG*, *ANK1*, *MKNK2*, *SPTB*, *ADA*, *C7* and *EPB420*. The role of these genes should be more explored in order to validate them as genetic markers to select against FHS/FHN, as well as improving the information available regarding the etiology of these conditions in chickens and possibly in humans.

Keywords: femoral head necrosis, RNA Seq, chicken, FHS

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LISTA DE SIMBOLOS E ABREVIATURAS

%	<i>percent</i>
°C	graus Celsius
µg	micrograma
AC	<i>articular cartilage</i>
AG	<i>affected group</i>
BP	<i>biological processes</i>
CBO	Condronecrose Bacteriana com Osteomielite
CC	<i>cellular components</i>
CG	<i>control group</i>
Cm	centímetros
DE	<i>differentially expressed</i>
DNA	<i>deoxyribonucleic acid</i>
g	gramas
GATK	<i>Genome Analysis Tool Kit 3.0</i>
GEO	<i>Gene Expression Omnibus</i>
GP	<i>growth plate</i>
kg.	Kilogramas
LogFC	<i>log2 fold-change</i>
MDS	<i>multidimensional scaling</i>
MF	<i>molecular functions</i>
MI	microlitro
Mm	milímetro
mRNA	RNA mensageiro
NCF	Necrose da Cabeça do Fêmur
ORA	<i>enrichment analysis</i>
pb	pares de bases
QC	quality control
RIN	<i>RNA integrity number</i>
RNA	<i>ribonucleic acid</i>
RNA-seq	sequenciamento de RNA
RT	<i>room temperature</i>
SNP	<i>single nucleotide polymorphism</i>
VEP	<i>Variant Effect Predictor</i>

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1. INTRODUÇÃO

A avicultura teve um crescimento exponencial nas últimas décadas, devido ao grande investimento em tecnologias e pesquisas, trazendo benefícios para a indústria e produtores (VOILÁ e TRICHES, 2013). A carne de frango é uma das proteínas mais consumidas no mundo, devido ao baixo custo e por ser considerada uma carne saudável e isenta de impedimentos culturais (VALCESCHINI, 2006). Para atender às demandas do mercado global é necessário garantir o bem-estar animal e a sustentabilidade de todo o processo. O constante investimento em melhoramento genético, nutrição, manejo e biosseguridade tem gerado um melhor desempenho dos frangos de corte, com a redução da idade de abate, melhor conversão alimentar e menor consumo de água, garantindo sempre o bem-estar animal (PIMENTEL, 2004).

Estima-se que nos últimos 50 anos as taxas de crescimento dos frangos tenham aumentado em mais de 300%, passando de 25g por dia para aproximadamente 100g por dia (KNOWLES et al., 2008). O melhoramento genético trouxe inúmeros benefícios, porém trouxe algumas consequências negativas, como problemas locomotores, distúrbios imunológicos e metabólicos (BARUT et al., 2008; TALLENTIRE et al., 2016), devido à seleção para crescimento rápido com formato corporal que atende às demandas industriais, maior musculatura torácica e redução da gordura abdominal.

Os problemas locomotores causam graves impactos na avicultura e afetam cerca de 6% dos animais em lotes comerciais, o que resulta em grandes perdas econômicas (PAZ, 2008). Além disso, animais com distúrbios locomotores têm seu bem-estar afetado, pois não conseguem se alimentar e beber água adequadamente, sendo suscetíveis a agentes oportunistas (BRADSHAW et al., 2002). Vários problemas locomotores afetam as aves, entre eles podemos citar: discondroplasia tibial, osteoporose, raquitismo, necrose da cabeça do fêmur, pododermatite, doença articular degenerativa, rotação tibial e síndrome dos dedos tortos (LIMA et al., 2007). A necrose de cabeça de fêmur (FHN), também conhecida como condronecrose bacteriana com osteomielite (BCO), é o distúrbio locomotor mais prevalente que

acomete frangos de corte (BRADSHAW; SAGE, 2001). A real incidência de FHN em frangos de corte é de difícil detecção, uma vez que as lesões muitas vezes permanecem subclínicas e sua base etiológica não é totalmente compreendida (PACKIALAKSHMI et al., 2015). A FHN ocorre na parte proximal da cabeça do fêmur das galinhas, iniciando com a degeneração da cartilagem articular e da placa de crescimento, causando a separação da cartilagem do osso, geralmente entre a região metafisária do osso e a placa de crescimento, causando a separação da cartilagem da cabeça do fêmur (FHS), também conhecida como epifisiolise (PACKISLAKSMI et al., 2015). Isso ocorre devido às altas taxas de crescimento e à deficiência de mineralização dos condrócitos que causam danos ao osso, permitindo a colonização por bactérias oportunistas, agravando o quadro (WIDEMAN; PRISBY, 2013).

As novas metodologias genômicas têm ajudado a aprimorar os programas de seleção genética de frangos por meio da seleção genômica. Também, a identificação de genes que controlam características relacionadas à integridade óssea e possíveis marcadores relacionados a doenças que impactam na produção e reduzem o bem-estar dos frangos, trazendo um grande avanço para as pesquisas que poderão ser aplicadas na indústria (PEIXOTO et al., 2020). Estudos estão sendo conduzidos para descobrir genes com papel potencial no desencadeamento de FHS e que podem causar BCO na cabeça do fêmur e na tíbia (PEIXOTO et al., 2019; HUL et al., 2020; PETRY et al., 2018; PALUDO et al. al., 2018; PALUDO et al., 2016; OLIVEIRA et al., 2020). Portanto, com o objetivo de investigar os eventos moleculares que estão envolvidos na FHS e subsequente FHN, este estudo buscou a identificação de genes diferencialmente expressos (DE) e processos biológicos compartilhados entre a cartilagem e a placa de crescimento da cabeça do fêmur de frangos de corte normais e afetados com FHS por meio do sequenciamento de RNA.

2.REFERENCIAL TEÓRICO

2.1. AVICULTURA ALIADA A SUSTENTABILIDADE E BEM-ESTAR ANIMAL

O setor avícola brasileiro teve um grande crescimento e fortalecimento nas últimas décadas e lidera a exportação mundial de carne de frango com 4,41 mil toneladas exportadas em 2019 (ABPA, 2020). Este grande desenvolvimento se deve aos constantes investimentos em toda a cadeia, como a tecnificação dos sistemas, uma maior organização e coordenação dos elos entre a indústria e produtores e também aos avanços obtidos através das pesquisas (VOILÁ e TRICHES, 2013).

A carne de frango é uma das proteínas mais consumidas no mundo, pois é livre de impedimentos culturais, tem um baixo custo e é considerada saudável por conter baixos teores de sódio e gordura (VALCESCHINI, 2006). O mercado sempre se molda às exigências dos consumidores: além da procura por alimentos mais saudáveis, a oferta de produtos seguros e sustentáveis é outra tendência de mercado (DIAS, 2016).

Para o Brasil, um dos maiores produtores e exportadores mundial de carne de frango, garantir a sustentabilidade do processo é essencial para a manutenção no mercado (DIAS, 2016). Para isso, é necessária uma produção com alta eficiência e baixo custo, permitindo que o animal expresse seu máximo potencial genético em um ambiente adequado e que tenha seu bem estar assegurado (RODRIGUES, 2014; PETRACI e CAVANI, 2012). O bem estar animal é essencial para se obter bons resultados na produção animal, e pode ser definido como “estado de harmonia entre o animal e seu ambiente, caracterizado por condições físicas e fisiológicas ótimas e alta qualidade de vida do animal” (HURNIK, 1992). Ele pode ser avaliado através de parâmetros como taxa de mortalidade, incidência de doenças, sucesso reprodutivo, comportamentos anômalos, presença de danos físicos entre outros (MENCH, 1993).

O investimento em melhoramento genético, nutrição e manejo resultou em um melhor desempenho do frango de corte, com a redução da idade de abate, melhor conversão alimentar e menor consumo de água, assegurando assim a sustentabilidade do processo, com um menor impacto para o meio ambiente (PIMENTEL, 2004). Porém, surgiram algumas consequências negativas como a

redução da imunidade das aves e aumento na ocorrência de problemas locomotores (HÖTZEL et al., 2004). Pensando nisso, a pesquisa aliada com as demandas da indústria, através dos programas de melhoramento genético vêm avaliando novos fenótipos e buscando alternativas viáveis para reduzir a incidência de condições que afetem a produção e o bem-estar animal (HELLMEISTER FILHO et al., 2003; PEIXOTO et al., 2020). A ferramenta de melhoramento genético e algumas de suas consequências serão discutidas no tópico seguinte

2.2. MELHORAMENTO GENÉTICO E SUAS CONSEQUÊNCIAS

A seleção genética é uma ferramenta que vem sendo usada desde o final da década de 1940, buscando melhorar os índices zootécnicos das aves, o que resultou no desenvolvimento da indústria avícola aumentando sua capacidade de produção (HAVENSTEIN; FERKET; QURESHI, 2003). Estima-se que nos últimos 50 anos, as taxas de crescimento dos frangos aumentaram mais de 300%, passando de 25g para cerca de 100g por dia (KNOWLES et al., 2008).

Devido à crescente demanda por carne de frango e pressão do mercado, os animais foram selecionados quanto aos parâmetros de produção (taxa de crescimento e conversão alimentar) e também quanto a conformação corporal, buscando animais com a musculatura de peito maior e redução da gordura abdominal (BARBUT et al., 2008). Devido ao rápido crescimento e ganho muscular, algumas aves podem desenvolver problemas locomotores, pois o peso vivo vai de 40g a 4kg em apenas 8 semanas, afetando a integridade estrutural do esqueleto (WIDEMAN; PRISBY, 2013; LEDUR et al., 2007).

Para minimizar esses problemas, as empresas de melhoramento genético têm aumentado a intensidade de seleção para critérios relacionados a robustez das aves (MUIR et al., 2014). Atualmente, avaliações com tomografia computadorizada tem permitido avanços na mensuração de fenótipos relacionados a qualidade das pernas nos frangos sem a necessidade de abater os animais (SOUZA, 2019).

Avanços recentes nas tecnologias de biologia molecular permitiram pesquisas para a identificação de genes específicos e marcadores de DNA envolvidos na resistência e susceptibilidade a doenças em aves (EFSA, 2010). Hoje, já é possível

realizar a genotipagem em grande escala dos animais, simultaneamente para dezenas de milhares de marcadores de DNA, permitindo assim a avaliação e seleção genômica, selecionando aves que naturalmente apresentem polimorfismos desejáveis (MEUWISSEN et al., 2001).

Dentre as doenças desenvolvidas ou agravadas devido a seleção genética, os distúrbios locomotores ganham destaque, pois afetam cerca de 6% dos animais em lotes comerciais, causando grande impacto devido ao aumento de condenação de carcaças e redução de produtividade (PAZ, 2008). Vários problemas locomotores afetam as aves, dentre eles podemos citar: a discondroplasia tibial, osteoporose, raquitismo, espondilolistese, síndrome da perna torta, necrose da cabeça do fêmur, pododermatite, doença articular degenerativa, rotação da tíbia e síndrome dos dedos tortos. Devido à dificuldade de locomoção, os animais não se alimentam e nem bebem água adequadamente, causando uma subnutrição que leva a uma queda de imunidade (GONZALES; MENDONÇA JUNIOR, 2006).

A doença locomotora com maior incidência nos frangos de corte é a Necrose da Cabeça do Fêmur, em inglês Femoral Head Necrosis (FHN), também conhecida como Condronecrose Bacteriana com Osteomielite, em inglês bacterial chondronecrosis with osteomyelitis (BCO) (BRADSHAW; SAGE, 2001). Essa afecção parece ser desencadeada pela separação da cartilagem da cabeça fêmur (FHS), em inglês femoral head separation (FHS) (PACKIALAKSHMI et al., 2015). O conhecimento do desenvolvimento dos tecidos ósseo e cartilaginoso ajuda no esclarecimento dos mecanismos de desencadeamento da FHS e posterior FHN, e será discutido no tópico a seguir.

2.3. DESENVOLVIMENTO ÓSSEO E DO TECIDO CARTILAGINOSO

A maioria dos estudos sobre FHS aborda somente o desenvolvimento ósseo, não dando a importância devida para a cartilagem articular, que também faz parte da articulação femoral. A cartilagem articular da cabeça femoral e a placa de crescimento óssea, local de proliferação celular do osso, diferem em relação à sua composição de histoarquitetura de matrizes extracelulares (ECM) e da capacidade de sofrerem hipertrofia (PACKIALAKSHMI et al., 2015).

A placa de crescimento óssea fica entre a epífise do osso, que está ligada a cartilagem, e a metáfise (Figura 1). O crescimento longitudinal dos ossos longos depende de uma placa de crescimento funcional, onde uma série de processos convergem para a proliferação de condrócitos e matriz celular (SIEBLER et al. 2001).

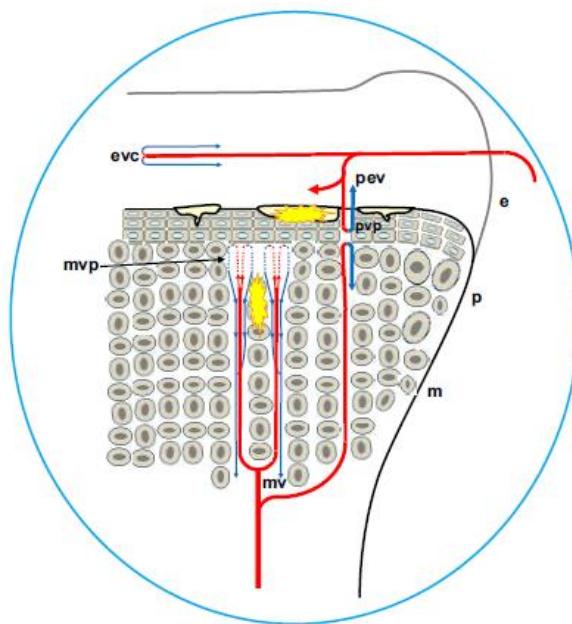


Figura1. Desenho representativo da cabeça proximal do fêmur, com a formação de fendas osteocondríticas na delimitação entre a epífise (e) e a placa de crescimento (physis, p). Os vasos metafisários ascendentes (mv) penetram através dos canais entre longas colunas de células calcificantes na metáfise (m). O plexo vascular metafisário (mvp) é formado por curvas nos capilares metafisários fenestrados que retornam como vênulas correndo pelo mesmo canal (setas azuis). As bactérias translocadas se espalham hematogenicamente e podem sair através do endotélio fenestrado nas pontas do plexo vascular metafisário, aderindo diretamente à matriz da cartilagem, colonizam fendas osteocondríticas e zonas de necrose e formam embolia obstrutiva na vasculatura metafisária.

Fonte: WIDEMAN; PRISBY, 2013.

O osso é considerado um tecido dinâmico, formado por osteoblastos e osteoclastos, e está em um processo contínuo de remodelação óssea, que ocorre durante toda a vida do animal (DIRCKS et al, 2019). O desenvolvimento ósseo se dá pela condrogênese, que inicia na vida embrionária, onde as cartilagens são

substituídas por ossos nas placas epifisárias, principalmente pós eclosão (DIBNER, et al. 2007).

O tecido cartilaginoso tem um papel importante na formação e proteção dos ossos, pois ele tem a função de suporte e revestimento de superfícies articulares, absorvendo choques e facilitando o deslizamento, sendo essencial na formação e crescimento dos ossos longos. O tecido cartilaginoso não possui vasos sanguíneos, por isso, é nutrido por capilares do pericôndrio ou através do líquido sinovial. Quando lesionado, ele se regenera com dificuldade e, na maioria das vezes, de modo incompleto (CAMANHO, 2001; LANZER; KOMENDA, 1990; JUNQUEIRA; CARNEIRO, 2008).

Os frangos têm um rápido período de formação óssea e mineralização, que ocorre do dia 4 ao dia 18 de vida e estima-se que o crescimento longitudinal dos ossos ocorra até 25 semanas de vida. Porém, a maioria das aves de corte comerciais são abatidas antes desta idade e, por consequência, antes de atingir a maturidade óssea necessária para suportar as altas taxas de crescimento dos frangos de corte modernos. Isso justifica a alta incidência de problemas locomotores observada nas últimas décadas (DIBNER et al. 2007; WILLIAMS et al. 2004; PINES et al. 2015).

2.4 NECROSE DE CABEÇA DE FEMUR (FHN)

A necrose de cabeça de fêmur (FHN), é uma importante causa de problemas locomotores em frangos de corte comerciais. A FHN se desenvolve na parte proximal cabeça do fêmur, iniciando com a degeneração da cartilagem articular e da placa de crescimento, ocasionando a separação da cartilagem do osso (Figura 2), o que predispõe a infecção bacteriana secundária (JIANG et al., 2015).



Figura 2. Fêmur normal (A) e afetado em frango com FHS (B). (PETRY et al., 2018)

Alguns fatores são apontados como predisponentes da FHN, como as altas taxas de crescimento e a deficiência de mineralização dos condrócitos que causam danos ao osso, e também fatores ambientais como manejo, idade, nutrição, e genética (KEALY, 1987; WIDEMAN; PRISBY, 2013). Em casos mais graves ocorre a contaminação por bactérias, que chegam através dos vasos sanguíneos (Figura 1), caracterizando a BCO (WIDEMAN; PRISBY, 2013). Algumas bactérias oportunistas como *Staphylococcus* spp, *Escherichia coli* e *Enterococcus* spp. foram isoladas das lesões de animais acometidos por BCO, sugerindo que as lesões encontradas nos ossos favorecem a sobrevivência destas bactérias aumentando a associação com a progressão da BCO (JIANG et al., 2015).

Os animais acometidos apresentam sérios problemas locomotores, afetando de forma uni ou bilateral (KEALY, 1987). Devido a isso, as aves ficam com dificuldade para se alimentar e beber água e o caso se agrava com o aumento da idade (GONZALES; MENDONÇA JÚNIOR, 2006). Apesar de alguns trabalhos estimarem a ocorrência dos problemas locomotores em aproximadamente 6 % de animais em lotes comerciais (PAZ, 2008), a real incidência de FHN não é conhecida, pois a maioria dos casos é subclínica.

2.5 ESTUDO DO TRANSCRIPTOMA E TÉCNICAS DE ANÁLISE DE RNA

O conhecimento da sequência total do genoma da galinha possibilitou que estudos de genômica funcional fossem realizados, esclarecendo a expressão e função dos genes de acordo com as células, tecidos, órgãos e seus mecanismos. Assim é possível, conhecer os transcriptomas correspondentes ao conjunto total de genes transcritos e começar a entender como podem ser regulados os genes codificadores e não codificadores de proteínas, proporcionando conhecimento sobre os mecanismos moleculares (DIAS, 2007, WANG et al., 2009).

Os perfis de um transcriptoma em resposta a estímulos fisiológicos e biológicos mostram informações valiosas para a interpretação da funcionalidade do genoma, revelando os mecanismos biológicos envolvidos (TANG et al., 2010). Diferentes tecnologias foram desenvolvidas para entender e quantificar o transcriptoma, podendo ser por meio de hibridização ou sequenciamento (WANG et al., 2009).

A análise de *Northern blotting*, uma das primeiras técnicas desenvolvidas para análise de RNA, permite que o RNA mensageiro (mRNA) de genes candidatos seja identificado por meio da hibridização de suas sequências (HE; GREEN, 2013). O mRNA é inicialmente separado por eletroforese em função de seu tamanho, sendo transferido para uma membrana, que é então hibridizado com sondas que são utilizadas para localizar o mRNA de interesse (THOMAS, 1983). Esta técnica é eficaz para detectar os transcritos, pois determina a abundância e os tamanhos dos transcritos de interesse. No entanto, se a quantidade de RNA total for limitada e o nível de expressão do transcrito for baixo, outras técnicas mais sensíveis são recomendadas (HE; GREEN, 2013).

Outra técnica amplamente utilizada principalmente nos anos 2000 foram os microarranjos (*microarrays*), também conhecidos como chips ou sondas, que consistem na hibridização de arranjos de DNA ou RNA que se encontram ordenados e distribuídos em uma superfície sólida, onde são marcados com moléculas fluorescentes permitindo posterior detecção proporcional ao número de moléculas hibridizadas. Porém, esta técnica se limita a leitura de transcritos conhecidos e contidos no chip (ROSA et al., 2007).

Por fim, o método de sequenciamento de RNA (RNA-seq), um dos mais utilizados atualmente, é uma técnica que possibilita a análise de todo o transcriptoma, pois é capaz de sequenciar tudo o que está sendo expresso em um determinado tecido, em um momento específico sem a necessidade de conhecimento prévio dos genes (TANG et al., 2010; WANG et al., 2011). Assim, o sequenciamento do mRNA possibilita descobrir novos genes, quantificar a expressão gênica, facilitando a anotação e montagens de novos transcritos, a identificação de sítios de *splicing* e a identificação de polimorfismos (SUNKARA; JIANG; ZHANG, 2012; TRAPNELL et al., 2012). Com isso, a partir da comparação de transcriptomas é possível compreender como as mudanças na atividade celular podem interferir em uma determinada doença (OKAZAKI et al., 2002).

2.6. ESTUDOS DE BIOLOGIA MOLECULAR E PROBLEMAS LOCOMOTORES

As novas metodologias genômicas têm ajudado a melhorar os programas de seleção genética dos frangos (DIAS et al., 2007). A identificação de genes que controlam características relacionadas a integridade óssea, e possíveis marcadores relacionados a doenças que causam impacto na produção e reduzem o bem estar dos frangos de corte, trazem um grande avanço para a indústria e pesquisa avícola (PEIXOTO et al., 2020). A identificação de genes e vias envolvidas com a FHN, juntamente com a rede de interações gênicas, é uma peça chave para elucidar a etiologia desta doença e também estratégias de prevenção através da seleção de aves mais robustas e ajustes de manejo e nutrição (PEIXOTO et al. 2019).

Trabalhos já publicados apontam genes com regulação negativa associados a FHN, como o fator de transcrição Runt 2 (*RUNX2*), osteonectina (*SPARC*) e fator de crescimento de fibroblastos (*FGF-1*), que têm um papel importante na vascularização e formação óssea (LI et al. 2015; PALUDO et al. 2017). Genes envolvidos na osteogênese (*ADIPOQ*, *COL8A1* e *SFRP5*), formação de tecido conjuntivo e produção de colágeno (*COL14A1*, *COL8A1* E *ANGPTL7*) também foram regulados negativamente em frangos de corte afetados por FHN (PETRY et al., 2018).

A maioria dos estudos avaliam apenas a expressão dos genes contidos no osso, e quase não existe informação sobre o que acontece no tecido cartilaginoso.

Com objetivo de realizar uma análise abrangente da FHS/FHN, o presente estudo buscou identificar o perfil de expressão dos genes e processos biológicos compartilhados entre esses dois tecidos, relacionados à FHS e subsequente FHN em galinhas e, também, possíveis marcadores genéticos que possam ser utilizados para a seleção de aves mais resilientes.

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3. CAPÍTULO I

Artigo submetido a revista Genomics

Comprehensive transcriptome analysis identifies core genes related to femoral head separation pathogenesis in chicken

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Abstract

Background

The Femoral Head Separation (FHS) is usually a subclinical condition characterized by the detachment of articular cartilage from the bone. This condition is closely related to Femoral Head Necrosis (FHN), one of the main locomotor problems that affect the poultry production. Therefore, in this study, a comprehensive analysis identifying shared expression profiles, biological processes (BP) and variants related to FHS and subsequent FHN in chickens was performed through RNA sequencing analysis. To this, two datasets containing 8 normal and 8 FHS-affected transcriptomes from broilers femoral articular cartilage (AC) and growth plate (GP) were analyzed. Furthermore, we have compared the differential gene expression found in FHS-chickens with other datasets available in public databases, trying to clarify shared genes and biological processes related to FHN in different species. A total of 36 differentially expressed (DE) genes were shared between AC and GP tissues, where more than 90% was upregulated in the FHS-affected broilers. Out of those, 23 genes were enriched in BP related to ion transport, translation factors and immune response. When the chicken DE genes were compared to other FHN datasets, 8 genes were shared among the evaluated species with normal and FHN affected samples: *SLC4A1*, *EPB42*, *ANK1*, *SPTB*, *CCL26*, *ADA*, *SLC25A37* and *MKNK2*. This study found DE genes between normal and FHS-affected broilers common to both analyzed tissues, showing shared mechanisms involved with this condition in chickens. Comparing the AC and GP DE genes with other datasets related to FHN allowed us to identify genes that are possibly involved with FHN across different species. We were able to find strong candidate genes related to FHS and FHN in chickens, such as *SLC4A1*, *RHAG*, *ANK1*, *MKNK2*,

SPTB, ADA, C7 and *EPB420*. The role of these genes should be more explored in order to validate them as genetic markers to select against FHS/FHN, as well as improving the information available regarding the etiology of these conditions in chickens and possibly in humans.

Keywords: gene expression, femoral head necrosis, inflammatory response, integrated analysis

Introduction

The poultry industry has experienced huge growth in recent decades, due to the advances in new researches and technologies which brought benefits to industry and producers [1]. Chicken meat is one of the most consumed proteins in the world, due to the low cost and for being considered a cost-effective healthy and exempted from cultural impediments [2]. The constant investment in genetic improvement, nutrition and management has generated a better performance of the broilers, with slaughter age reduction, better feed conversion and less water consumption, always ensuring the animal welfare [3].

The genetic breeding programs have brought numerous benefits for poultry production, such as the increased body weight, greater thoracic musculature and reduction of abdominal fat. In the last 50 years, the chicken growth rates have raised more than 300%, from 25g per day to approximately 100g per day [4,5]. However, some negative consequences, such as locomotor problems, immunological and metabolic disorders have appeared due to the selection for fast-growing [5,6].

Locomotor problems cause severe impacts on the poultry industry affecting about 6% of the animals in commercial flocks, which results in economic losses [7]. Furthermore, animals with bone disorders have their welfare affected because they cannot eat and drink water properly, being susceptible to opportunistic agents [8]. The bacterial chondronecrosis with osteomyelitis (BCO), also known as femoral head necrosis (FHN) is the most prevalent leg disorder in broilers [9]. The real incidence of FHN in broilers is difficult to estimate since most of the lesions often remain subclinical and its etiological basis is not fully understood [10]. However, some authors have shown 20% of FHN incidence in chickens [11] FHN occurs in the proximal part of the chickens' femur head, starting with the femoral head separation (FHS), which consist in the separation of the growth plate from the articular cartilage [10,12]. This occurs due to the high growth rates and the mineralization deficiency of the chondrocytes that cause damage to the bone, which could allow the colonization by opportunistic bacteria featuring the BCO [13].

After decades of selection, the body weight of broilers increase significantly, changing the center of gravity and increase the mechanical stress at the femur growth plates [11,14]. FHS is consider a metabolic skeletal problem in fast-growing chickens that increase the vulnerability of the femoral joint [15]. Since the etiology of FHN remains unclear, studies are being conducted to clarify its pathogenesis, the molecular mechanisms involved and to discover genes with a potential role in triggering FHS and subsequent BCO in the head of the femur and tibia [11,16–20]. Although most of the previous studies describe biological processes (BP) involved with this condition in bone tissue, there are no studies evaluating gene expression profiles between bone and articular cartilage of normal and FHN-affected chickens. Therefore, in this study, the

femoral growth plate (GP) and articular cartilage (AC) transcriptomes from normal and FHS-affected broilers we compared using RNA sequencing to identify common molecular mechanisms and differentially expressed genes (DE) between both tissues, and polymorphisms highlighting possible new candidate genes involved with FHS in chickens.

Material and Methods

Animals and sample collection

The Embrapa Swine and Poultry National Research Center Ethical Committee of Animal Use (CEUA/CNPSA) approved this study under protocol number 012/2012. Commercial male broilers (Cobb500) with 35 days of age from a poultry farm located in Concórdia, Santa Catarina State, Brazil, were used as described by Peixoto et al. (2019) and Hul et al. (2020). Briefly, 29 broilers (14 normal and 15 with lameness) were selected and sent to the Embrapa Swine and Poultry National Research Center for sample collection. The proximal femoral head was classified based on the clinical examination of the separation of the growth plate (GP) from the articular cartilage (AC), according to Wideman et al. (2012). The normal (control) group (CG) was characterized by good adhesion between the GP and AC, and the FHS-affected group (AG) presented the separation between the GP and AC. After the group characterization between CG and AG, the femoral AC and GP were collected, stored in liquid nitrogen and transferred to a freezer at -80°C until the samples were processed as described by Peixoto et al. (2019) [16] and Hul et al. (2020) [21].

RNA extraction, library preparation and sequencing

For RNA extraction, 100mg of GP and AC tissue samples were macerated in liquid nitrogen, adding 1 mL of the Trizol reagent (Invitrogen, USA), vortexing and incubating for five minutes at room temperature (RT). For GP samples, the RNA extraction was performed, followed by an RNA cleanup using RNeasy mini kit (Qiagen. Germany) according the manufacturer's instructions, as described by Peixoto et al. (2019). For AC samples, the procedure was described by Hul et al. (2020). RNA quantification was performed with Biodrop (Biodrop. Cambridge. UK) spectrophotometer and quality was assessed with Agilent 2100 Bioanalyzer (Agilent. California. USA) and through visual inspection of 1% agarose gel. Only samples with RNA integrity number (RIN) higher than 8 were selected, resulting in a total of 16 samples (4 AC normal, 4 AC affected, 4 CG normal and 4 CG affected) used to construct the RNA-seq libraries. For GP, the libraries were prepared from 2 µg of RNA using the "TruSeq™ RNA Sample Prep Kit v2" (Illumina. Inc.; CA. USA), while for AC the "TruSeq Total Stranded Sample Preparation" (Illumina. Inc.; CA. USA) kit was used. The library size ($200\text{pb} \pm 25\text{ pb}$) was quantified by qPCR and evaluated in Bioanalyzer (Agilent) before sequencing. Libraries were submitted to the Functional Genomics Center, ESALQ, University of São Paulo, Piracicaba, São Paulo State, Brazil, for sequencing in Illumina HiSeq2500 equipment (Illumina. Inc.; San Diego. CA. EUA), following the 2x100bp paired-end protocol, with samples of each tissue in a different lane. The fastq files used in this study were obtained from BioProject numbers PRJNA350521 for AC and PRJNA352962 for GP, f, generated by Peixoto et al. (2019) and Hul et al (2020), respectively, and previously deposited in the SRA database.

Quality control, mapping, differential expression analyses and functional annotation

Quality control (QC) was performed with the Trimmomatic tool [22] to remove short reads (<70pb), low-quality reads (QPhred <20) and adapter sequences. The data quality was visualized using the FastQC tool [23] [23]. Sequence reads for AC and GP were mapped against the chicken reference genome (GRCg6a) with the Ensembl release 101 (www.ensembl.org) using the STAR software [24]. The reads were also counted with STAR [25], which uses the HTSeq count algorithm considering the exon regions. The EdgeR package [26] from R (R Core Team. 2013) was used to identify the differentially expressed (DE) genes between the control (CG) and affected (AG) groups for each tissue separately. Genes with false discovery rate (FDR) <0.05 were considered DE, after correcting for the Benjamini-Hochberg (BH) multiple-tests [27]. The genes were considered upregulated and downregulated according to the positives and negatives log₂ fold-change (LogFC) respectively, in the affected compared to normal samples. R software was used to create a multidimensional scaling (MDS) plot with the LogFC values from each gene and a heatmap based on DE genes to check the consistency between. A smear plot was also created using R to highlight the results from the differential expression test. The expressed genes were annotated using the Biomart database (<https://www.ensembl.org/biomart>). The David 6.8 [28] and Panther (<http://pantherdb.org/>) databases were used performing gene ontology analysis of DE genes, to obtain the biological processes (BP), cellular components (CC), molecular functions (MF) and metabolic pathways. The enrichment analyses were performed using the chicken genome information available in those databases.

Integrated analysis

To evaluate the main mechanisms involved in FHS, the NetworkAnalyst [29] was used to perform a meta-analysis across the AC and GP datasets. Once the common genes were obtained, the David 6.8 [28] database was used to find the shared biological processes involved with FHS based on the gene ontology database. The Revigo tool [30] was used to reduce and highlight the most abundant BP in our data. A gene network with the common DE genes between the two tissues was obtained using the String database tool [30].

Furthermore, to better understand the main genes involved with FHS across different species. we have performed a search in the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/gds>) using the terms femoral head necrosis and epiphysiolysis. This search returned two main datasets: Identification of Potential Biomarkers for Improving the Precision of Early Detection of Steroid-induced Osteonecrosis of the Femoral Head (GSE123568) and Gene expression profiling of hip cartilage with necrosis of femoral head (GSE74089). The first one evaluated the gene expression on blood from 40 human patients (10 normal and 30 affected with femoral head necrosis) and the second one, the hip cartilage in 24 human patients (12 normal and 12 affected with femoral head necrosis). Using the GEOR tool, also from the GEO dataset (<https://www.ncbi.nlm.nih.gov/geo/info/geo2r.html>), the DE genes were obtained. The list of DE genes from the two datasets obtained in the GEO database and common DE genes found in the GP and AC from our study were submitted to the InteractiVenn (<http://www.interactivenn.net/>) to verify the common DE genes across all experiments.

Polymorphism identification using the RNA-Seq data

The Genome Analysis Tool kit 3.6 (GATK) [27] was used for polymorphism identification. We have followed the best practices guidelines standard parameters for transcriptome variant analysis available at the GATK website (<https://gatk.broadinstitute.org/>). The Picard tools 2.5 (<https://broadinstitute.github.io/picard/index.html>) was used to generate the reference genome, the genome index, to assign read groups and mark duplicates. In the GATK, the CIGAR strings determination (Split'N'Trim), qualities reassessing mapping, base recalibration, variants calling (HaplotypeCaller) and filtering were performed. To minimize the false positive variants, the following filters were used in GATK ? to filter and select variants: SNP cluster 3 variants in a 35bp window, FS > 30.0, QD < 5.0, MQ < 50.0, MQRankSum < -12.5, ReadPosRankSum < -8.0, GQ < 5.0, QUAL ≥ 30.0 and DP ≥ 100.0. Furthermore, the final dataset was filtered out and the variants with less than 10 reads per sample were removed from the downstream analysis. Once the polymorphisms were identified in the GP and AC datasets, a filter was performed to obtain the variants that differed between normal and FHS-affected groups. The Variant Effect Predictor (VEP) tool [28] was used for variant annotation, effect and consequence predictions using the *Gallus gallus* genome (GRCg6a) with the Ensembl 103 annotation database version. Furthermore, the STRING and EnrichR [31] databases were used to verify the interactions between DE genes and variants in genes identified.

Results

RNA-Sequencing, mapping and characterization

The RNA sequencing of all samples ($n = 16$) generated approximately 345 million paired end reads and after the quality control analyses remained 306 million reads (88.78%) remained for further analyses (Additional file 1: Tble S1), with an average of 16,355,490 reads for GP and 26,819,697 for AC. A separation between the samples from the two tissues, as well as, between the normal and affected groups can be verified in the heatmap and MDS plot figures (Additional file 2: Fig S1 and Fig S2, respectively). As expected, based on the type of library preparation, most of the genes were characterized as protein-coding (95%), and the remaining 5% were classified in *lncRNA*, pseudogenes, miRNAs, and other noncoding RNAs (Table 1).

Table 1 Transcripts characterization identified in the articular cartilage (AC) and femoral growth plate (GP) tissues

Annotated transcripts	AC	GP
IG_V_gene	3	0.02%
LncRNA	413	3.40%
MiRNA	20	0.16%
misc_RNA	1	0.01%
mt_RNA	2	0.02%
protein_coding	11577	95.03%
Pseudogene	155	1.27%
rRNA	1	0.01%
scaRNA	1	0.01%
snoRNA	8	0.07%
snRNA	1	0.01%
sRNA		1
Total annotated transcripts	12182	12632

Common differentially expressed genes in AC and GP transcriptomes

Using the Ensembl annotation 101, in the AC transcriptome, 12,182 genes were identified, where 106 genes were DE: 99 (93.44%) were upregulated and 7 (6.56%) were downregulated in the AC of the FHS-affected compared to normal group (Additional file 1: Table S2). Regarding the GP tissue, 12,632 genes were expressed, where 324 were DE between the analyzed groups. From those, 174 were upregulated (53.7%) and 150 (46.3%) downregulated in the affected compared to the control group (Additional file 1: Table S3).

Comparing the DE genes between the articular cartilage (AC) and the growth plate (GP), 36 DE genes were common in both transcriptomes (Table 2). From these 36, 33 were upregulated and 3 downregulated in the FHS-affected group in both transcriptomes.

Table 2. Common differentially expressed genes between articular cartilage (AC) and femoral growth plate (GP) with information of the Ensembl ID, gene name, \log_2 fold change (logFC) and false discovery rate (FDR).

Ensembl ID	Gene name	AC		GP	
		logFC	FDR	logFC	FDR
ENSGALG00000043254	<i>EPX</i>	4.25	4.11E-09	2.59	0.0009
ENSGALG00000003212	<i>TSP02</i>	3.66	7.20E-05	1.54	0.005
ENSGALG00000040279	<i>RHCE</i>	2.02	0.0008	1.29	0.002
ENSGALG00000026518	<i>RUNDC3A</i>	2.44	0.003	1.10	0.02
ENSGALG00000039978	<i>SLC4A1</i>	2.70	0.003	1.20	0.01
ENSGALG00000007447	<i>TUBB1</i>	1.99	0.003	1.28	0.01
ENSGALG00000011190	<i>PLACL2</i>	2.54	0.005	1.10	0.0002
ENSGALG00000042105	<i>PLCB2</i>	1.81	0.005	0.67	0.03
ENSGALG00000014585	<i>CCL26</i>	2.36	0.008	1.12	0.04
ENSGALG00000021230	<i>EPB42</i>	2.69	0.008	1.33	0.02
ENSGALG00000004170	<i>ADA</i>	1.71	0.008	1.01	0.001
ENSGALG00000015358	<i>MYH15</i>	-1.91	0.01	-1.38	0.03

ENSGALG00000016684	<i>RHAG</i>	2.43	0.01	1.34	0.006
ENSGALG00000029857	<i>GIMAP6</i>	1.79	0.01	0.76	0.01
ENSGALG00000013575	<i>IFI6</i>	2.87	0.01	2.22	0.01
ENSGALG00000009479	<i>SAMD9L</i>	3.10	0.01	1.78	0.01
ENSGALG00000000378	<i>SLC25A37</i>	1.76	0.01	1.32	0.001
ENSGALG00000041693		2.81	0.01	1.33	0.04
ENSGALG00000003845	<i>MKNK2</i>	1.40	0.01	0.63	0.009
ENSGALG00000026948	<i>ADD2</i>	2.12	0.01	1.27	1.85
ENSGALG00000036805	<i>SPTB</i>	2.19	0.01	1.09	0.005
ENSGALG00000051068		2.38	0.02	1.41	0.0005
ENSGALG00000003594	<i>ANK1</i>	2.11	0.02	0.99	0.01
ENSGALG00000014736	<i>KEL</i>	1.99	0.02	1.21	0.005
ENSGALG00000043671		-1.24	0.02	-1.03	0.02
ENSGALG00000028273	<i>HBE1</i>	3.74	0.02	2.24	0.04
ENSGALG00000039269	<i>RNF213</i>	2.06	0.027	1.06	0.03
ENSGALG00000014835	<i>C7</i>	2.84	0.027	1.55	0.001
ENSGALG00000028357	<i>LOC428421</i>	2.50	0.036	1.49	0.006
ENSGALG0000002192	<i>PTPRC</i>	1.22	0.037	0.61	0.03
ENSGALG00000045776	<i>CPN2</i>	1.77	0.037	0.90	0.02
ENSGALG00000014686	<i>FBN2</i>	-1.08	0.037	-1.72	0.0005
ENSGALG00000030247	<i>TMOD4</i>	2.05	0.04	1.06	0.01
ENSGALG00000009552	<i>LOC423277</i>	1.78	0.04	1.14	0.04
ENSGALG00000044326	<i>LOC426820</i>	1.54	0.04	0.80	0.01
ENSGALG00000006453	<i>TF</i>	3.04	0.04	0.67	0.04

A total of nine main biological processes were described in the articular cartilage DE genes response to biotic stimulus, ion transport, immune system process, interspecies interaction between organisms, iron ion homeostasis, cytolysis, muscle contraction, myofibril assembly and peptide cross-linking (Additional file 2: Figure S3 and Table S4). In the bone, 11 main biological processes were enriched, such as response to calcium ion, bone trabecula formation, heterocycle metabolic process, iron ion homeostasis, heme biosynthetic process, cholesterol import, cell adhesion, response to stimulus, immune system process, interspecies interaction between organisms and protein heterotrimerization (Additional file 1: Figure S4 and Table S5). When the enrichment analysis was performed based on the common DE genes, the

main biological processes were found related to ion transport, inflammatory and defense response, homeostasis, protein biogenesis and immune response processes (Fig 1).

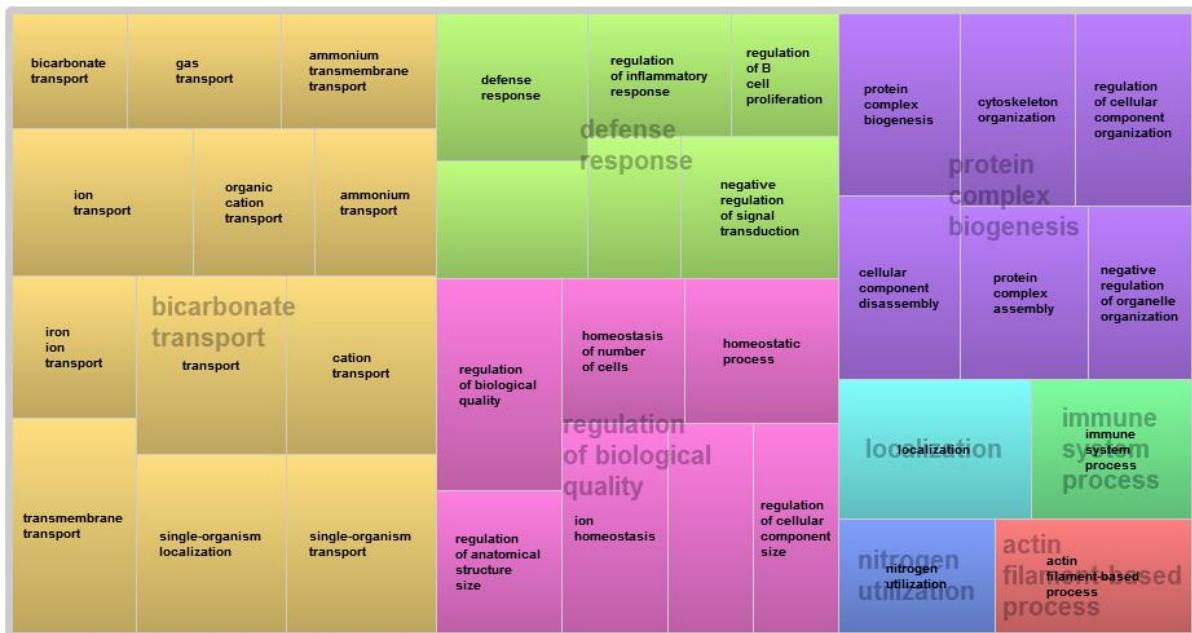


Fig 1. Main biological processes involved with the DE genes shared between articular cartilage and femoral growth plate using the REVIGO tool.

A gene network was constructed based on the 36 common DE genes. From those, 23 were recognized by STRING tool, where it was possible to observe a main network grouping genes related to ion transport and translation factors (Fig 2). The *TF* gene is involved in antimicrobial activity, while some genes such as *HBGS* and *RHAG* are involved in oxygen and ions transport. Another group connected the *PTPRC*, *CCL10*, *ISG12-2* and *SAMD9L* genes that are most related to the immune response BP (Fig 2).

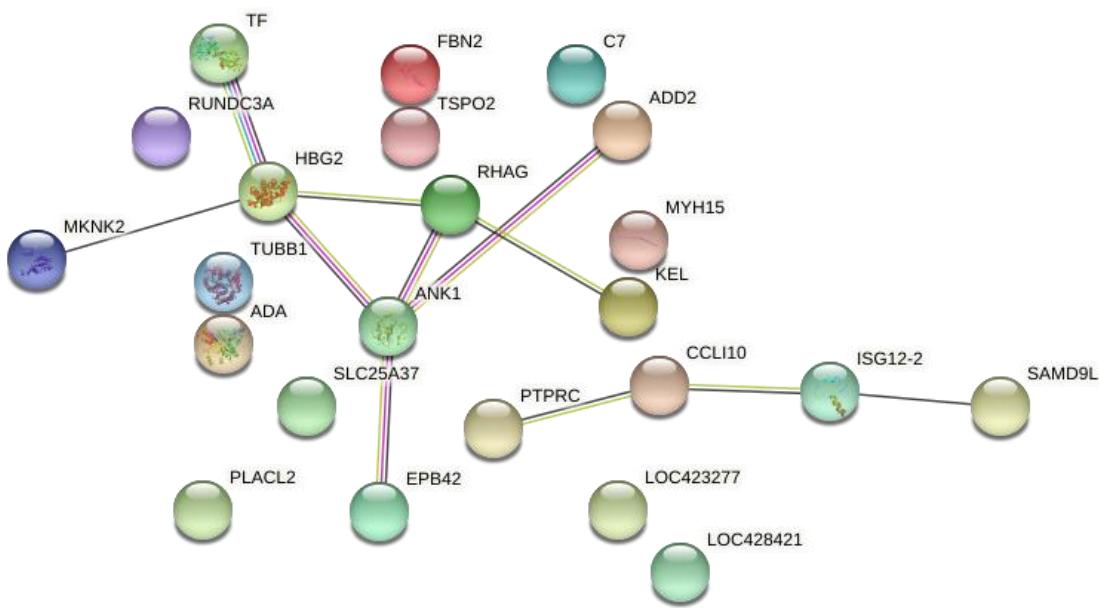


Fig 2. Gene network of the common differentially expressed genes between the AC and GP tissues from normal and FHS-affected groups using STRING. Coloured circles represent genes and lines represent the predicted interactions between genes.

Comparison of the DE genes of AC and GP tissues with different datasets

After the differentially expressed analysis, we compared the DE genes from the AC and GP tissues with other datasets obtained in the GEO database, as previously described in the Material and Methods section. According to the GEOR analysis, the comparison performed between normal and FHN-affected individuals (GSE123568) evinced a total of 5250 DE genes in the blood of those individuals, while 5978 DE genes were found in the hip cartilage of normal and FHN-affected individuals (GSE74089). Considering these four datasets, it was possible to observe that four genes were DE in all studies: *SLC4A1* (Solute Carrier Family 4 Member 1), *EPB42* (Erythrocyte Membrane Protein Band 4.2), *SPTB* (Spectrin Beta, Erythrocytic) and *ANK1* (Ankyrin 1) (Fig 3), all of them related to erythrocyte and ion transport functions.

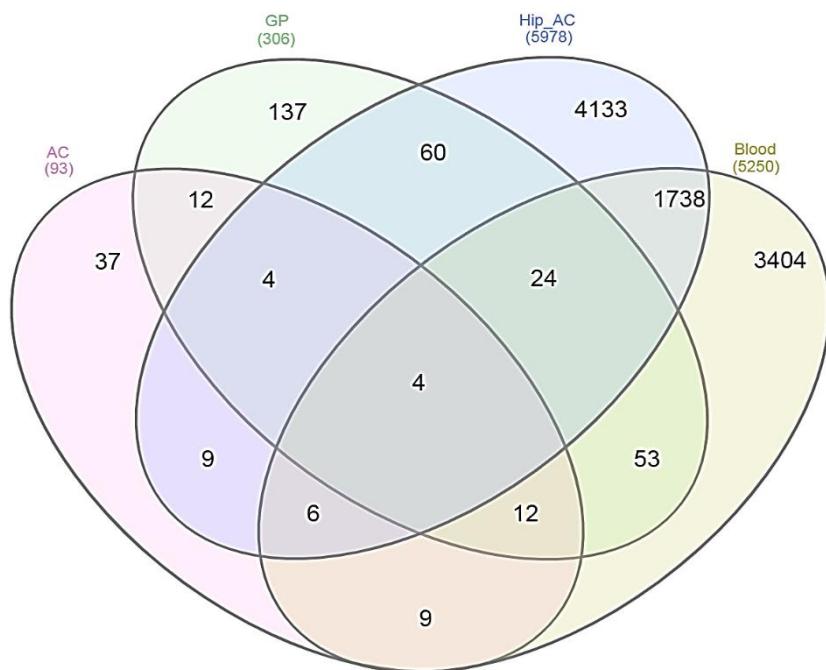


Fig 3. Venn diagram from DE genes from normal and femoral head necrosis and/or FHS-affected samples in the 4 evaluated datasets: AC: chicken femoral articular cartilage; GP: chicken femoral growth plate; Hip_AC: human articular cartilage and Blood: human peripheral blood.

We have also performed a comparison using DE genes from cartilage (chicken and human) and bone datasets to verify the presence of shared genes in the tissues affected by FHN/FHS. In this comparison, eight shared genes were found, the four previously described (*SLC4A1*, *EPB42*, *ANK1* and *SPTB*) and the genes four additional genes: *CCL26* (Chemokine (C-C motif) ligand 26), *ADA* (Adenosine Deaminase), *SLC25A37* (Mitoferin-1) and *MKNK2* (MAPK Interacting Serine/Threonine Kinase 2), all involved with the immune response (Fig 4).

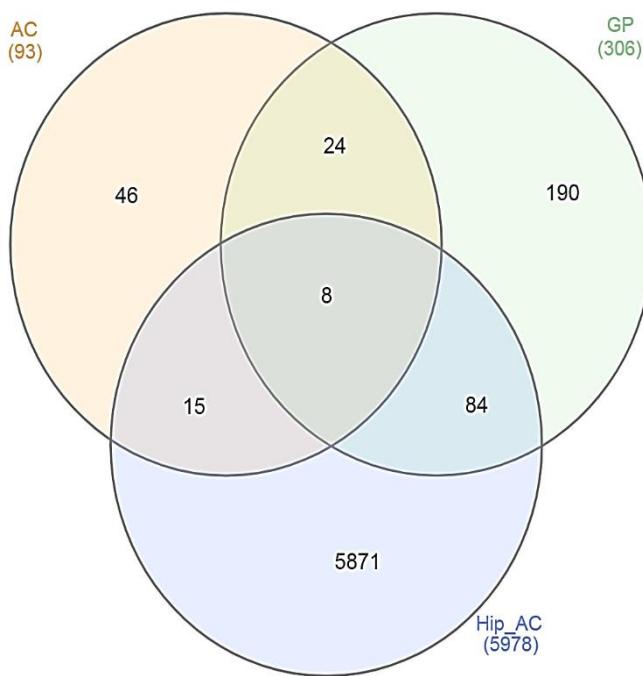


Fig 4. Venn diagram from DE genes from normal and femoral head necrosis and/or FHS affected samples in the 3 evaluated datasets: AC: chicken femoral articular cartilage; GP: femoral growth plate; Hip_AC: human articular cartilage.

Discussion

The mechanical stress caused by the overload to the skeleton that modern chickens sustain due to the high growth rates has been recognized as one of the main causes of locomotor problems [30-33]. Among these conditions, the FHS and posterior FHN/BCO are disorders that has a large impact on the poultry industry due to the huge economic losses and its negative effect on animal welfare. One of the biggest issues to study these conditions is the early detection of FHS/FHN, since animals do not show clinical signs often, being visible only after slaughter [33].

There are few studies related to FHS/FHN and other bone integrity problems especially those approaching the molecular mechanisms involved with these conditions. Previous studies of our group have shown some biological processes (BP) and genes associated with FHN in chickens in different lines and ages [16–18,20]. Paludo et al [19] pointed out that the downregulated expression of *RUNX2* and *SPARC* genes may be associated with reduced vascularization and poor bone mineralization, increasing the risk of skeletal problems in chickens. The impaired collagen formation, connective tissue cell adhesion and extracellular bone matrix (ECM) were also identified as predisposing factors for FHN/BCO and other leg disorders [16–18,20]. Also, the translocation of bacteria from blood to bone has been suggested as a secondary condition [20], whereas the bacterial profile of microbiome in the blood of animals affected with BCO was different from the healthy ones [34]. However, most of these studies focusing only on the bone tissue, and furthermore, there are no studies evaluating the articular cartilage and the femoral head jointly. Therefore, our study focused to find common genes, BP and variants in the AC and GP of broilers normal and affected with FHS through an integrated analysis of the RNA sequencing datasets previously published [16,17].

In the global transcriptome characterization of the GP and cartilage AC tissues about 12,000 genes were expressed in both tissues, where 11,141 were shared and 891 and 441 genes were exclusively from GP and AC respectively. As expected, due to the RNA sequencing methodology used, approximately 95% of the genes were coding, although IncRNAs, miRNAs, mitochondrial and miscRNAs were also expressed in the analyzed datasets (Table 1). Considering the DE analysis, 106 genes were DE in AC, where 93.44 % of the were upregulated in FHS-affected animals

(Additional File 1: Table S1), while in the GP, 324 DE genes were obtained, only 53.7 % was upregulated in the AG when compared to the normal ones (Additional File 1: Table S2), results that were very similar to the obtained by Peixoto et al. (2019) and Hul et al. (2020) [16,17].

Out of the DE genes observed in AC and GP tissues, 36 DE genes were shared by both tissues. It is interesting to note that 91.6% of the common genes were upregulated in the FHS-affected group (Table 2). Some of those genes have already been highlighted in other studies [16–18]. However, when the common BPs were evaluated were related to ion transport, defense response and those related with tissue homeostasis (Fig 1)

Two main branches were observed in the gene network (Fig 2), where one was composed by the *TF*, *HBG2*, *ANK1*, *SLC25A37*, *EPB42*, *RHAG*, *ADD2*, *KEL*, *MKNK2* and another by the *PTPRC*, *CCLI10*, *ISG12-2 AND SAMD9L*. In general, the first group of genes are related to blood type, related to cartilage regeneration and inflammatory response [16,35].

In addition to the identification of the DE genes, biological processes and SNPs found in this study, another interesting result found here is related to the comparisons of the chicken transcriptomes with other datasets available in the GEO database. When we compared the DE genes obtained in the AC and GP tissues with other gene expression datasets obtained in the GEO, we found four genes in common, *SLC4A1* (Solute Carrier Family 4 Member 1), *EPB42* (Erythrocyte Membrane Protein Band 4.2), *SPTB* (Spectrin Beta. Erythrocytic) and *ANK1* (Ankyrin 1) (Figure 3), all of them related to erythrocyte and ion transport functions. In the second comparison, using the DE

genes of bone and cartilage datasets, besides the four previously described (*SLC4A1*, *EPB42*, *ANK1* and *SPTB*), four additional genes (*CCL26*, *ADA*, *SLC25A37* and *MKNK2*, all involved to immune response. For a better understanding of the function of the highlighted genes, we have grouped according to the main BPs that will be discussed below.

Homeostasis, ions and erythrocyte transport

Homeostasis is a constant process of the organism to be balanced, where the ions exchange and erythrocyte transportation have an important role. In this study, these processes were one the main BPs to be highlighted. These BP are also involved in the control of bone growth by blood vessels, through angiogenesis and angiotropic signals mediating the transport of circulating cells, oxygen, nutrients and waste products [36].

The *ANK1* gene encodes a protein related to the binding of the structural constituent of the cytoskeleton, helping to binding of other membrane proteins to the actin-spectrin cytoskeleton [37]. It also acts on activation, proliferation, contact and maintenance of specialized membrane domains [37]. Multiple ankyrine isoforms with different affinities for various target proteins are expressed in a tissue-specific manner, regulated by development. The *ANK1* is usually found in erythrocytes, but it has already been found in the brain and muscles [38]. Mutations in the *ANK1* gene were associated to an increase in the osmotic cell fragility and in a reduction in the stability of ANK1 proteins [39]. These results were also related to reduction of this in the plasma membrane where the interaction with SPTB and SLC4A1 occurs [39]. In our study, the high expression of *ANK1* maybe can alter the structure of the actin cytoskeleton, affecting the structural integrity of the articular cartilage of the femur, contributing to

the occurrence of proximal femoral epiphysiolysis. In addition, its expression is related to cell damage, which can be a consequence of FHS, when epiphysiolysis starts, positive regulation can act as a sign of attempt to combat the progression of this condition. This gene is also involved in the upregulation of inflammatory cytokines in osteoarthritic lesions [40]. The *ANK1* gene is regulated by the EPB42, another gene that was upregulated in the FHS-affected group.

The *transferrin (TF)*, which was in the main branch of the gene network, primary function is to transport iron from storage and adsorption sites to tissues requiring iron [41]. This gene has an effective line of defense against systemic infection by withholding iron from invading microbes [41]. There are two forms of *transferrin* in birds, *ovotransferrin*, found in oviducts, and serum transferrin, secreted by the liver. Serum transferrin may also play a role in stimulating cell proliferation and is regulated by iron levels, while *ovotransferrin* has a bacteriostatic function and is not controlled by iron levels [42,43]. Furthermore, the *TF* has an important role in the endochondral ossification, being considered the angiogenic molecular released by the hypertrophic cartilage [44] . The *HBG2* (*Hemoglobin Subunit Gamma 2*) is involved in the transport of oxygen from the lungs to the peripheral tissues, where the beta chain is a component of adult hemoglobin A and D [45]. This gene, as the *TF*, was upregulated in the FHS-affected animals (Table 2). The gamma globin genes (*HBG1* and *HBG2*) are normally expressed in the fetal liver, spleen and bone marrow (*HBG2*. GENE CARDS;2020). Chicken has multiples types of globins and the erythropoiesis occurs in two waves: one primitive that giving rise to blood cells during early embryonic development, and a second that produces definitive erythrocytes during late embryonic and post hatching

development [47]. In the bone and cartilage, its function is not clear, as well as, no information is available regarding its in FHS in chickens.

The *MKNK2* (Serine / threonine-protein kinase 2) gene interacts with MAP kinase, and may play a role in the response to environmental stress and cytokines. Among its related pathways are the interleukin 1 signaling pathway. MAP/ERK signaling and it acts as a mediator of the suppressive effects of IFN-gamma in hematopoiesis [48]. The *MKNK* is not normally expressed in rats, but appears in stressful situations, showing its adaptive function and can also be a signal for cellular apoptosis [49]. Bringing it to our study, the gene may be involved in the initial phase of FHN with the function of initial signaling.

Blood circulation, inflammatory and immune response

Another BP observed is the blood circulation that is linked to the immune response and inflammation. When tissue damage or inflammation occurs, there is an increase in blood flow, capillary permeability and an influx of immune cells to repair tissue. The *MYH15* has also been associated with the adrenergic signaling process in cardiomyocytes, in cases of imbalance in the O₂ supply and removal of CO₂ from tissues [50]. This gene could be associated to FHS as a consequence of local inflammation.

The *EPX* gene is activated during the immune response, releasing proteins and other components that have a toxic effect on damaged cells or invading organisms [51]. One of the released proteins is the eosinophil peroxidase, which is extremely cytotoxic and has anti-inflammatory and pro-inflammatory properties, regulating

inflammation by fighting invading microorganisms [52,53]. In chickens, the *EPX* gene has been studied as a biomarker for inflammatory events in the gastrointestinal tract [54] and has been related to leg disorders [16,17]. The *EPX* upregulation in broilers affected with FHS may indicate the immune system response to inflammation and, in severe cases, may be related to local necrosis.

The extracellular matrix (ECM) is a stable structural component that is located under the epithelium and surrounds connective tissue cells [55]. The ECM is responsible for providing support and resistance to tissues and organs throughout the body and acts in the body in biochemical processes that will assist in the morphogenesis, differentiation and homeostasis of tissues [56]. The upregulated genes *ADA* and *RHAG* also participate in the process of metabolic glycosaminoglycans (GAGs) and aminoglycans involved in the metabolism of ECM [57], indicating that the body tries to repair the damage caused by FHS through tissue remodeling. Furthermore, the *ADA* enzyme that acts as an endogenous regulator of the adaptive immune system, with an emphasis on the proliferation and differentiation of T lymphocytes, regulates cell metabolism and triggers a variety of physiological effects on cell proliferation [58,59].

The inflammation is an essential component of the immune system; however, the excess of inflammation can cause tissue damage [60]. The *ADA* gene also acts as a sensor providing information to the immune system about tissue damage, protecting host cells from excessive tissue damage associated with inflammation [61]. The upregulation of *ADA* can be related to a regulatory role in immune responses. acting on the activation and regulation of lymphocyte and neutrophil levels [59,62]. In our study, the *ADA* was the upregulated in AC and GP in the FHS-affected group (Table

2), possibly due to the large tissue inflammation that occurs in the affected animals. The presence of specific variants in this gene between the studied groups also highlights that genetic mechanisms could be involved in FHS in chickens.

The *ADA* and *IFI6* genes also play an important role in the regulation of apoptosis, which is an essential physiological mechanism in the development and tissue homeostasis (*ADA* - GeneCards ; Petry et al., 2018). The *IFI6* gene, also known as *ISG12*, regulates cellular metabolism during the differentiation of osteoblasts and apoptosis [29]. The upregulation of the *IFI6* gene may be related to a causative factor, stimulating apoptosis in the articular cartilage, leading the animal to be more susceptible to FHN.

In addition to the identification of the DE genes, biological processes and SNPs found in this study, another interesting result found here is related to the comparisons of the chicken transcriptomes with other datasets available in the GEO database. When we compared the DE genes obtained in the AC and GP tissues with other gene expression datasets obtained in the GEO, we found four genes in common, *SLC4A1* (Solute Carrier Family 4 Member 1), *EPB42* (Erythrocyte Membrane Protein Band 4.2), *SPTB* (Spectrin Beta. Erythrocytic) and *ANK1* (Ankyrin 1) (Figure 3), all of them related to erythrocyte and ion transport functions. In the second comparison, using the DE genes of bone and cartilage datasets, besides the four previously described (*SLC4A1*, *EPB42*, *ANK1* and *SPTB*), four additional genes were found (*CCL26*, *ADA*, *SLC25A37* and *MKNK2*, all involved to immune response. Most of them have already been described previously, however, two genes, the *CCL26* and *SLC4A1* have not been enriched in the gene network. The *CCL26* (chemokine (C-C motif) ligand 26) is from the cytokine family of secreted proteins involved in immunoregulatory and

inflammatory processes, displays chemotactic activity for normal peripheral blood eosinophils and basophils and may contribute to the eosinophil accumulation in atopic diseases (*CCL26 - GeneCards*). Finally, the *SLC4A1* gene encodes a protein that is part of the anion exchanger family and is expressed in the plasma membrane of erythrocytes, involved in the transport of carbon dioxide from the tissues to the lungs (HAO et al., 2019). A major integral membrane glycoprotein of the erythrocyte membrane is required for normal flexibility and stability of the erythrocyte membrane (*GeneCards*). Furthermore, this gene is located in QTL regions previously described to femur mineral content and femur weight, which reinforces its importance as a good candidate gene to FHS.

Based on the functions and the high expression of the genes that were DE in both tissues, we had two hypotheses: the first is that the high expression of these genes may be a consequence of the FHS, due to cell damage and also the attempt to combat the progression of this condition, and the second hypothesis is that the high expression and possible mutations of these genes cause FHN, as they can alter the structure of the actin cytoskeleton, affecting the structural integrity of the femoral articular cartilage, contributing to the occurrence of FHS. Therefore, this study found DE genes between normal and FHS-affected groups shared with both analyzed tissues, showing common mechanisms involved with this condition in chickens.

Conclusions

Comparing the AC and GP DE genes with the other datasets related to FHN allowed us to identify s genes that are possibly involved with FHN, evincing the shared

mechanisms across different species. Finally, with the integrated analysis of gene expression and variant identification, we were able to find strong candidate genes related to FHS/FHN/BCO in chickens, and that the role of *SLC4A1*, *RHAG*, *ANK1*, *MKNK2*, *SPTB*, *ADA*, *C7* and *EPB420* genes should be more explored in order to validate them as molecular markers to FHS/FHN.

Declarations

Ethics approval: All animal procedures were performed in accordance with the Ethics Committee on Animal Utilization of the Embrapa Swine and Poultry National Research Center under protocol number 012/2012.

Consent for publication: Not applicable

Availability of data and material: The datasets used or analyzed during the current study are available from the corresponding author on reasonable request. The transcriptome sequences are available in the SRA database with BioProject number PRJNA352962 and PRJNA350521.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: AMGI, JOP and MCL conceived and designed the experiment. JOP and MCL were responsible for the data collection. MEC, AMGI and IG performed the RNA-Seq analysis. AMGI, MEC and IG performed the functional analyses of the genes. AMGI, JOP, MEC, IG and MCL interpreted the results and

evaluated the conclusions. AMGI and IG wrote the manuscript. All authors reviewed, edited and approved the final manuscript.

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Supplementary Information

Additional file 1: Table S1. Input paired read numbers, Reads number after quality control and mapping information of each sample. A table containing the number of raw reads and after quality control and also the mapping information. **Table S2.** Expressed and DE genes between normal and FHS-affected broilers with 35 days of age in the AC. An excel sheet with the expressed and DE genes between normal and FHS-affected broilers with 35 days of age. **Table S3.** Expressed and DE genes between normal and FHS-affected broilers with 35 days of age in the GP. An excel sheet with the expressed and DE genes between normal and FHS-affected broilers with 35 days of age. **Table S4.** GO biological process grouped in the Revigo tool based on the differentially expressed genes in the femoral articular cartilage (AC) of normal and FHS-affected broilers. **Table S5.** GO biological process grouped in the Revigo tool based on the differentially expressed genes in the femoral growth plate (GP) of normal and FHS-affected broilers.

Additional file 2: Fig S1. Heatmap gene cluster classification for AC and GP normal and FHS-affected samples. In the heatmap, the expression for each gene is presented in the rows and sample is visualized in the columns, showing a hierarchical clustering of genes and samples. In red, DE genes were downregulated and in green, upregulate in the affected samples. **Fig S2.** Multi-Dimensional Scaling Plot (MDS-plot) measuring the similarity of the samples into 2-dimensions (black represents the AC samples and red the GP samples). **Fig S3.** Superclusters of biological process enriched for up and downregulated genes related to FHS in AC obtained using the Revigo tool. Different colors show different superclusters and the size of each box is determined by the uniqueness of the categories. **Fig S4.** Superclusters of biological process enriched for up and downregulated genes related to FHS in GP obtained using the Revigo tool. Different colors show different superclusters and the size of each box is determined by the uniqueness of the categories.

Figure Legends

Fig 1. Main Biological process involved the DE genes shared between articular cartilage and femoral growth plate using the REVIGO tool.

Fig 2. Gene network of the common differentially expressed genes between the AC and GP tissues from normal and FHS-affected groups using STRING. Colored circles represent genes and lines represent the predicted interactions between genes.

Fig 3. Venn diagram from DE genes from normal and femoral head necrosis and/or FHS-affected samples in the 4 evaluated datasets: AC: chicken femoral articular

cartilage; GP: femoral growth plate; Hip_AC: human articular cartilage and Blood: human peripheral blood.

Fig 4. Venn diagram from DE genes from normal and femoral head necrosis and/or FHS affected samples in the 3 evaluated datasets: AC: chicken femoral articular cartilage; GP: femoral growth plate; Hip_AC: human articular cartilage.

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